AD_____

Award Number: W81XWH-04-1-0216

TITLE: BTG2 Antiproliferative Gene and Prostate Cancer

PRINCIPAL INVESTIGATOR: Paul D. Walden, Ph.D.

CONTRACTING ORGANIZATION: New York University School of Medicine
New York, NY 10016

REPORT DATE: February 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

☐ Distribution limited to U.S. Government agencies only; report contains proprietary information

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-02-2006	Annual	16-01-2005 – 15-01-2006

4. TITLE AND SUBTITLE

Antiproliferative Gene and Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0216

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Paul D. Walden, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

New York University School of Medicine
New York, NY 10010

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
and Materiel Command
Fort Detrick
Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES: Original contains colored plates: ALL DTIC reproductions will be in black and white

14. ABSTRACT Expression of the protein product of the BTG2 tumor suppressor is lost during the transition of normal prostate epithelial cells into prostate cancer cells. Restoration of BTG2 expression in BTG2 null prostate cancer cells significantly reduces cell proliferation and tumorigenicity. Steady state levels of BTG2 protein are regulated post-translationally in p53 positive prostate cells. Our working hypothesis being tested as part of our proposed studies is that the tumor suppressive activity of BTG2 is lost as an early event in prostate carcinogenesis due to increased proteasomal degradation, leading to compromised cell cycle regulation, increased cell invasion and cancer progression. To date we have shown that BTG2 protein expression is lost as a very early event in prostate cancer and that prostate cancer tissue and cells degrade BTG2 at a greater rate than non-cancer tissue and cells. In the present reporting period we have shown that the subcellular localization and degradation kinetics of BTG2 are markedly regulated during the cell growth cycle. Specifically BTG2 is predominantly nuclear consistent with its antiproliferative function, but also BTG2 becomes concentrated in the nucleolus localization 4 hours following growth stimulation with EGF indicating that BTG2 might additionally influence some aspect of ribosome biosynthesis.

15. SUBJECT TERMS
Tumor suppressor gene, prostate cancer, tumor biology

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION oF ABSTRACT: UU**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U		9	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

INTRODUCTION: The protein product of the B-cell translocation gene-2 (BTG2) antiproliferative gene acts at the G1 and G2/M checkpoints via Rb- and p53-dependent and independent mechanisms. We have shown that BTG2 acts in a cytoprotective manner to prevent malignant transformation of prostate epithelial cells [1,2]. Furthermore, forced expression of BTG2 in PC-3 prostate cancer cells (which do not normally express detectable levels of BTG2) is associated with significantly reduced rates of both cell proliferation and tumorigenicity [1,2]. $BTG2^{-/-}$ cells continue to proliferate in the presence of DNA damaging agents [3] predisposing cells to the accrual of genetic damage. Steady state levels of BTG2 protein are regulated post-translationally in p53 positive prostate cells and increased rates of proteasomal degradation may account for loss of BTG2 protein expression during prostate carcinogenesis [2]. Furthermore, we have shown that ectopic expression of BTG2 in BTG2 null PC-3 prostate cancer cells causes reduced expression of cyclin D1, cyclin B, and thymosin β4 indicative of a role in reducing cell migration as well as blocking cell cycle progression. Consistent with this finding**,** forced expression of BTG2 in PC-3 cells was associated with reduced local tumor growth and absence of metastases following orthotopic injection in the prostates of nude mice. These findings have provided the basis for formulating our working hypothesis that the tumor suppressive activity of BTG2 is lost as an early event in prostate carcinogenesis due to increased proteasomal degradation, leading to compromised cell cycle regulation, increased cell invasion and cancer progression. The purpose of this research is to test this hypothesis, and we proposed 3 tasks to accomplish this: Task 1: Define BTG2 expression during the morphological transition of normal prostate epithelial cells into prostate cancer precursors (PIA and HGPIN); Task 2: Compare the mechanism of BTG2 protein degradation in non-cancerous and cancerous prostate tissue and epithelial cells and Task 3: Determine the effects of BTG2 on prostate cell attachment and invasion.

BODY: Research accomplishments associated with the approved *Statement of Work* are described below. Only new research accomplishments since the previous report are described.

Task 1: Define BTG2 expression during the morphological transition of PIA (proliferative inflammatory atrophy) into HGPIN (high-grade prostatic intraepithelial neoplasia) (months 1-5).
All the studies in task 1 were completed and described in the previous annual report. There were no major changes to the approved Statement of Work.

Task 2: Compare the mechanism of BTG2 protein degradation in non-cancerous and cancerous prostate tissue and epithelial cells (months 5-26).
The majority of this task has been accomplished and the remainder of the studies will be completed in the final year of funding.

a. Histologically characterize and isolate adjacent regions of non-cancerous and cancerous prostate tissue (months 5-9).
This has been completed.

b. Determine the rates of BTG2 protein degradation in extracts of non-cancerous prostate tissue specimens and prostate cancer and determine the contribution of the ubiquitin-proteasome pathway to the degradation process (months 5-9).
This has been completed and the results were presented in the previous annual report.

c. Establish and maintain primary cultures of prostate epithelial cells and the LNCaP and PC-3 cell lines for study (months 10-26).
We are presently conducting these studies.

d. Determine the rates of BTG2 protein degradation in extracts of non-cancerous and cancerous prostate epithelial cells and prostate cancer cell lines and determine the contribution of the ubiquitin-proteasome pathway to the degradation process (months 10-14).
We have completed these studies. Extracts prepared from primary cultures of cancerous human prostate epithelial cells degrade BTG2 at a significantly greater rate than extracts from primary cultures of non-cancerous human prostate epithelial cells (Table 1).

Half-life, minutes		*P* value
Non-Cancerous	Cancerous	
27.3 ± 2.1	16.5 ± 1.2	0.0017

Table 1. Half–life of BTG2 protein in extracts of primary cultures of cancerous and non-cancerous human prostate epithelial cells

Rates of BTG2 protein degradation by extracts of LNCaP and PC-3 prostate cancer cells were similar to those in cancerous prostate epithelial cells. In all cell lines we were able to inhibit BTG2 degradation by addition of the proteasome inhibitor lactacystin. These findings suggest that the discrepancy between steady sate levels of BTG2 protein comparing cancerous and non-cancerous prostate epithelial cells is due at least in part to increased proteasomal degradation in cancer.

e. Determine the rates of BTG2 protein degradation during the cell-cycle in extracts of non-cancerous and cancerous prostate epithelial cells and prostate cancer cell lines (months 14-18).
We have completed now these studies, however, they took substantially longer than we had originally planned for several reasons. The major reason was due to technical difficulties with cell synchronization. Initially, the cells could not be reliably synchronized in the commercially available growth medium we typically use for epithelial cells (Keratinocyte SFM). They would not grow or synchronize at low densities necessary to conduct these studies. To resolve this issue we switched to the original “home-made” complex medium we had previously used to grow these cells [5]. After resolving these issues, we were able to get reproducibly synchronized cells by making them quiescent in the absence of EGF and allowing the cells to enter the growth cycle by addition of EGF. We were then able to get very reproducible BTG2 staining during the cell cycle using these conditions (Figure 1).

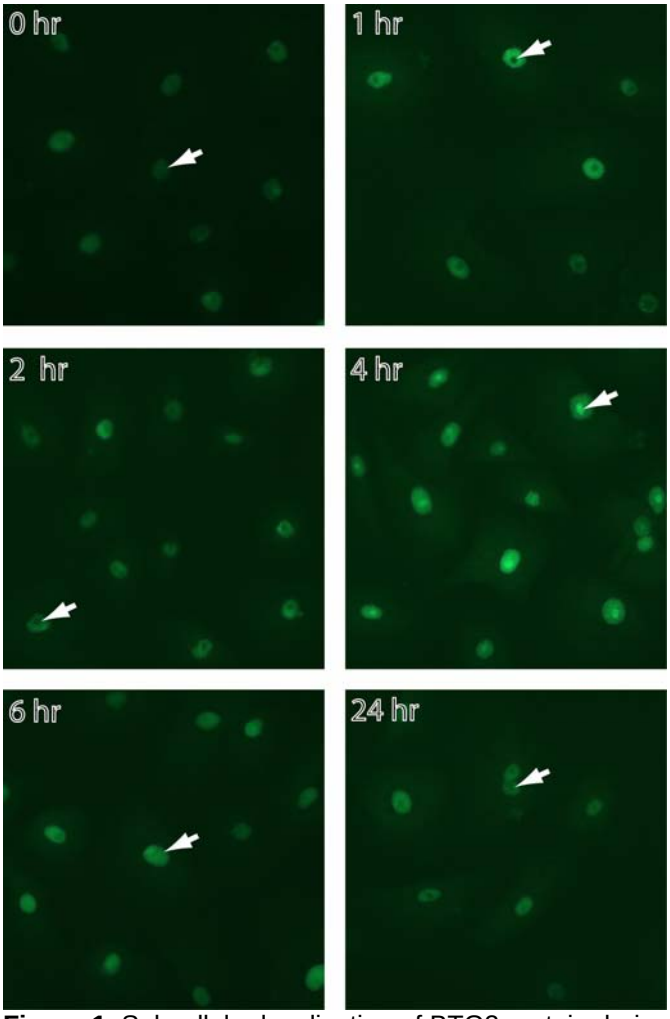

Figure 1. Subcellular localization of BTG2 protein during the cell cycle of primary cultures of non-cancerous human prostate epithelial cells. BTG2 protein was localized in by immunofluorescence microscopy in primary cultures of non-cancerous human prostate epithelial cells that were quiescent (0 hr) or had been stimulated to enter the growth cycle by addition of EGF (1 hr to 24 hr). Arrows show nucleolus.

BTG2 shows predominantly nuclear localization throughout the cell cycle, entirely consistent with its localization in human prostate and breast tissues. Interestingly and unexpectedly, prior to 4 hr BTG2 does not accumulate in the nucleolus, but at 4hr (corresponding with the S-phase of the cell cycle) there is intense nucleolar staining for BTG2. After 4 hours, until 12 hours BTG2 is localized throughout the nucleus and nucleolus in the majority of cells. After 12 hours, nucleolar staining decreases and by 24 hours nucleolar staining has diminished. The BTG2 localization pattern was very similar in cancer cells, except the signal was much weaker (data not shown). Thus differences in localization cannot account for differences in BTG2 function in cancer. In HGPIN, when BTG2 protein levels decline weak nucleolar staining for BTG2 persisted. Nuclear localization of BTG2 is consistent with its antiproliferative function, but given its nucleolar localization, BTG2 might influence some aspect of ribosome biosynthesis. While outside the scope of the present award, elucidating the nucleolar function of BTG2 will be a future goal of our laboratory.

It has long been known that BTG2 is antiproliferative in response to growth stimulation, but the mechanism remained elusive until recently. In the past year it was shown that BTG2 can induce apoptosis in response to EGF in U937 tumor cells via a p53 independent mechanism that involves phosphorylation of BTG2 by Erk1/2 which in turn induces BTG2 to bind to Pin-1[4]. BTG2 is therfore able to inhibit cell cycle progression and malignant transformation by multiple mechanisms, which is entirely consistent with our working hypothesis.

Studies on BTG2 protein degradation during the cell cycle are included in section f.

f. Determine if there are differential rates of BTG2 protein synthesis in addition to differential rates of protein degradation (months 18-23).
We have completed these studies. The rates of BTG2 protein synthesis and degradation during the cell cycle relative to rates in quiescent cells are shown in Figure 2.

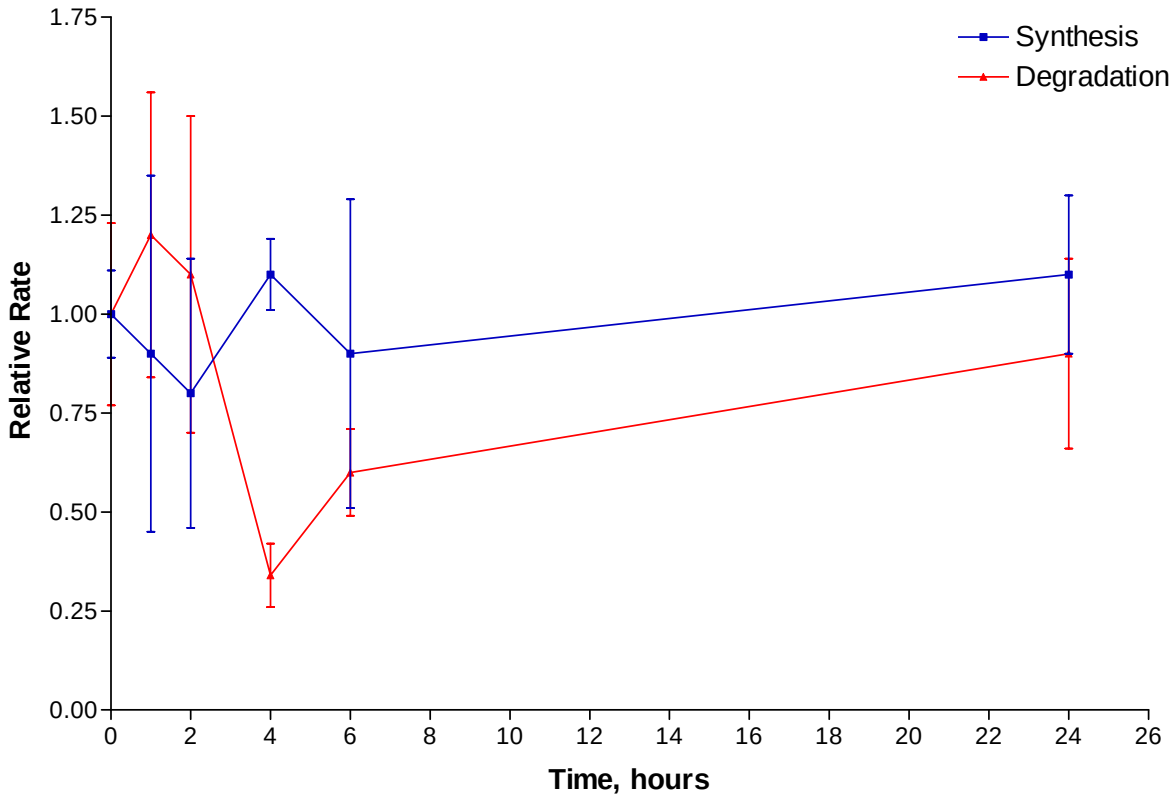

Figure 2. Rates of BTG2 protein synthesis and degradation in primary cultures of prostate epithelial cells. Rates of synthesis and degradation were determined by pulse-chase labeling with ^{35}S-methionine followed by immunoprecipitation with anti-BTG2 antibodies and polyacrylamide gel electrophoresis. Quantification was determined by phosphorimager analysis of the dried gel. Rates were determined relative to quiescent cells which were assigned the arbitrary value of 1.

Rates of BTG2 protein synthesis were not significantly different during the cell cycle, however rates of degradation were significantly reduced at 4 hr coincident with nucleolar localization of BTG2 (Figure 1). Similar results were seen in cancer cells although the reduced degradation at 4

hr was not as pronounced (data not shown). Thus are marked differences in the cell cycle dependent degradation and subcellular accumulation of BTG2.

g. Determine the extent of ubiquitination of BTG2 in extracts of non-cancerous and cancerous prostate epithelial cells and prostate cancer cell lines (months 23-25).
Due to the technical difficulties with cell synchronization mentioned earlier, these studies were initiated later than planned (month 24). We plan to complete these studies in the final year of funding.

h. Determine the levels of de-ubiquitinating enzyme Unp-1 in extracts of non-cancerous and cancerous prostate epithelial cells and prostate cancer cell lines (months 24-26).
Due to the technical difficulties with cell synchronization mentioned earlier, these studies were initiated later than planned (month 24). We plan to complete these studies in the final year of funding.

Task 3: Determine the effects of BTG2 on prostate cell attachment and invasion (months 26-36).
a. Maintain cultures of PC-3/Retro-Off BTG2 cells that express BTG2 under the control of an inducible tetracycline promoter (months 26-36).
b. Determine whether BTG2 expression in PC-3/Retro-Off BTG2 cells increases cell attachment to different extracellular matrices (months 26-31).
c. Determine whether BTG2 expression in PC-3/Retro-Off BTG2 cells decreases tumor cell invasion through the extracellular matrix (months 31-36).
We plan to initiate task 3 on schedule.

KEY RESEARCH ACCOMPLISHMENTS:

- Shown the half-life of BTG2 to be significantly reduced in cancerous vs non cancerous prostate epithelial cells.
- Shown that BTG2 is a nuclear protein throughout the cell cycle and that the protein accumulates in the nucleolus at 4 hr following entry into the cell cycle stimulated with EGF.
- Shown that BTG2 is synthesized at similar rates during the cell cycle, but that degradation is reduced at 4 hr following entry into the cell cycle stimulated with EGF.
- Taken in light of other published studies our studies support the concept that BTG2 is an endogenous cell death molecule which acts at multiple points in regulation of the cell cycle to inhibit the early phases of carcinogenesis in prostate, breast and kidney.

REPORTABLE OUTCOMES:

- We submitted a manuscript to Cancer Research this year that was not accepted. We will revise this manuscript to include the new data from this annual report that we will resubmit in 2006.
- We have generated reagents from cell cycle synchronized cancerous and non-cancerous prostate epithelial cells that are amenable to further study for continued study of the findings uncovered in the current period of research.

CONCLUSIONS: The research completed to date shows that the BTG2 tumor suppressor is lost as an early event in prostate carcinogenesis, and other studies have shown a similar pattern in breast and kidney tumors. Our research completed to date confirmed our working hypothesis that the reason for BTG2 protein loss in prostate cancer is due to increased proteasomal degradation of the protein. Studies from other investigators have shown that BTG2 can influence multiple aspects of cell cycle progression and malignant transformation. Studies from this year of support have additionally indicated a hitherto unknown function for BTG2 in the nucleolus. Loss of BTG2 has been shown to occur as an early event in carcinogenesis in other solid tumors and this may be an important general mechanism in carcinogenesis. Further elucidating the function of BTG2 is therefore critical to understanding the process of carcinogenesis. The goal of our final year of support is to study functional consequences of loss of BTG2 in prostate carcinogenesis.

REFERENCES:

1. Walden, P. D., Lefkowitz, G. K., Ficazzola, M., Gitlin, J., and Lepor, H. Identification of genes associated with stromal hyperplasia and glandular atrophy of the prostate by mRNA differential display. Exp.Cell Res., 245: 19-26, 1998.
2. Ficazzola, M. A., Fraiman, M., Gitlin, J., Woo, K., Melamed, J., Rubin, M. A., and Walden, P. D. Antiproliferative B cell translocation gene 2 protein is down-regulated post-transcriptionally as an early event in prostate carcinogenesis. Carcinogenesis, 22: 1271-1279, 2001.
3. Rouault, J.-P., Falette, N., Guehenneux, F., Guillot, C., Rimokh, R., Wang, Q., Berthet, C., Moyret-Lalle, C., Savatier, P., Pain, B., Shaw, P., Berger, R., Samarut, J., Magaud, J.-P., Ozturk, M., Samarut, C., and Puisieux, A. Identification of BTG2, an antiproliferative p53-dependent component of the DNA damage cellular response pathway. Nature Genet., 14: 482-486, 1996.
4. Hong, J.W., Ryu, M.S. and Lim, I.K. Phosphorylation of serine 147 of tis21/BTG2/pc3 by p-Erk1/2 induces Pin-1 binding in cytoplasm and cell death. J. Biol. Chem. 280:21256-21263, 2005.
5. Walden PD, Ittmann M, Monaco ME, Lepor H. Endothelin-1 production and agonist activities in cultured prostate-derived cells: implications for regulation of endothelin bioactivity and bioavailability in prostatic hyperplasia. Prostate. 34:241-50, 1998
6. Kawakubo, H., Carey, J. L., Brachtel, E., Gupta, V., Green, J. E., Walden, P. D., and Maheswaran, S. Expression of the NF-κB-responsive gene BTG2 is aberrantly regulated in breast cancer. Oncogene, 23:8310-8319, 2004.